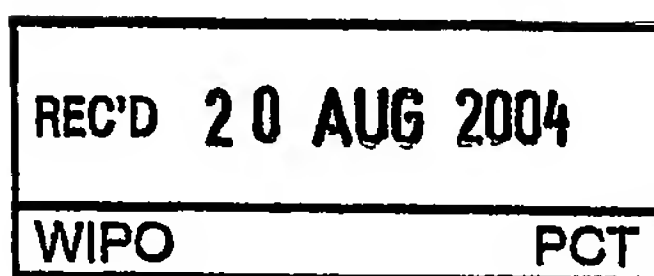


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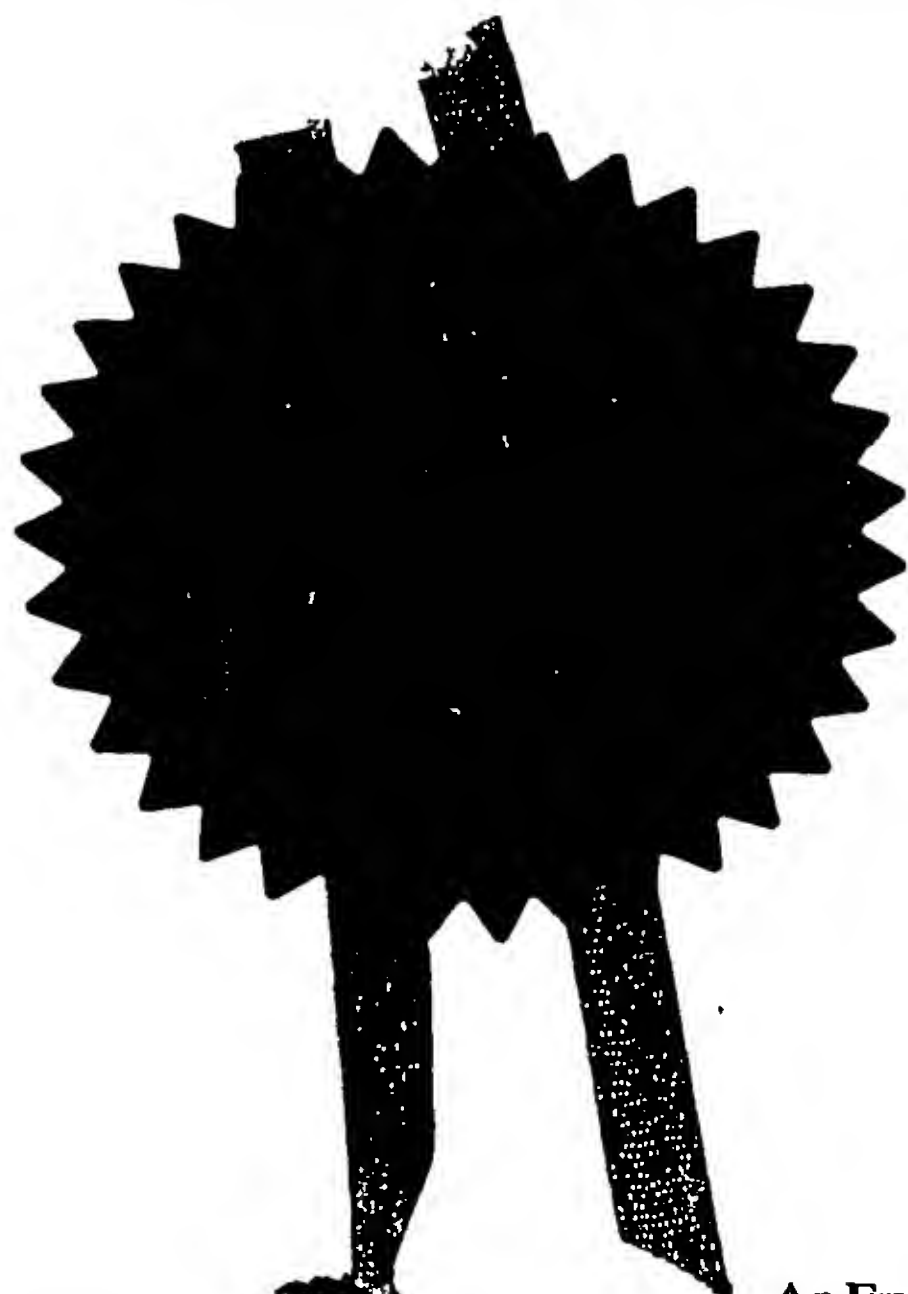
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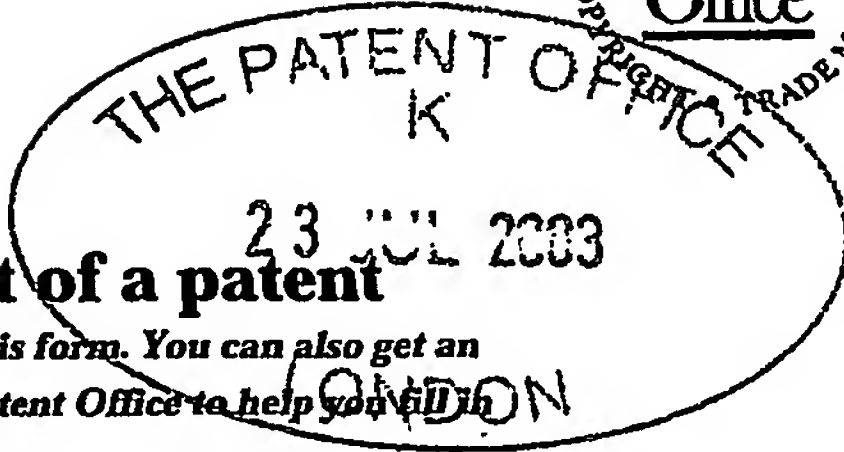
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Dated

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*R. Mahoney*



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N.86335 JCI

2. Patent application number

(The Patent Office will fill in this part)

0317240.0

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BIOCONTROL LIMITED  
Cranfield Innovation Centre, University Way  
Cranfield Technology Park  
Cranfield, Bedfordshire MK43 0BT

Patents ADP number (if you know it)

8413254001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

THERAPEUTIC AGENTS

5. Name of your agent (if you have one)

J.A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

14 South Square  
Gray's Inn  
London  
WC1R 5JJ

Patents ADP number (if you know it)

26001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

YES

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
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Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

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Request for substantive examination (*Patents Form 10/77*)

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(please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature

*J. A. Kemp & Co*  
J.A. KEMP & CO.

Date 23 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

J.C. IRVINE  
020 7405 3292

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## THERAPEUTIC AGENTS

### Field of the Invention

5

The present invention relates to therapeutic and diagnostic preparations comprising viruses that kill bacteria (bacteriophages). In particular, the invention relates to the use of such preparations as control agents for infections of animals and humans caused by pathogenic bacteria including *Pseudomonas aeruginosa*.

10

### Background to the Invention

15

Antibiotics have been seen for many years as "the answer" to the problem of bacterial infections. This attitude persisted until the development of the wide-ranging (and in some cases total) resistance to antibiotics seen within the last ten years. In many cases it is necessary to use expensive "drugs of last resort" (such as vancomycin for *Staphylococcus aureus*), which often require complex routes of administration and show toxic side effects, necessitating prolonged hospital treatment.

20

Even to these drugs, resistance is reaching worrying levels. It is now clear that bacteria can adapt to resist any antibiotic. Even the new generation drugs such as linezolid are already generating resistance [Mutnick *et al* (2003) *Ann Pharmacother* 37:769-774; Rahim *et al* (2003) *Clin Infect Dis* 36: E146-148], and it is clear from recent developments that resistance develops faster than new antibiotics can be produced, evaluated and processed through regulatory approvals.

25

A further disadvantage of antibiotic treatment is its lack of specificity. Antibiotics can kill a wide range of bacteria and this can lead to recolonisation of the body by inappropriate and often harmful bacteria. There is therefore a need for an antibacterial treatment that shows specificity against particular bacterial species so that little resistance is induced in the normal flora.

30

The need for new forms of antibacterial therapy is well illustrated by the case of infection with the Gram-negative aerobic bacterium *Pseudomonas aeruginosa*.

5 *Pseudomonas aeruginosa* is a serious opportunistic bacterial pathogen. Infections caused by *Pseudomonas aeruginosa* include:

- *Otitis externa* of dogs, a chronic disease of the ears, common in inbred (pedigree) dogs
- *Otitis externa* of humans (“swimmers ear”) along with other ear infections  
10 and other topical infections of humans including pseudomonas keratitis and pseudomonas folliculitis
- Infection of burns and skin grafts in humans
- Hospital-acquired infections
- Lung infection in cystic fibrosis (CF) patients

15

10-15% of nosocomial (hospital acquired) infections are due to *Pseudomonas aeruginosa*, with 2 million cases annually in the US alone. In some situations, the frequency is even higher. Of the 150,000 burn patients treated in US hospitals and burn centres per year, 26% have *Pseudomonas aeruginosa* infections. *Pseudomonas*  
20 *aeruginosa* is notorious for its resistance to antibiotics so infections caused by it can be difficult to treat. One of its natural habitats is soil, where it is exposed to organisms that produce antibiotics. This may well have led to the development of resistance mechanisms coded for both by genes on the chromosome and by transferable genetic elements known as *plasmids*. The properties of the *P.*  
25 *aeruginosa* outer membrane are important in conferring resistance. An additional resistance mechanism is its tendency to grow on available surfaces as complex layers [Donlan (2002) Emerging Infectious Diseases 8: 881-890,  
<http://www.cdc.gov/ncidod/EID/vol8no9/02-0063.htm>; Fletcher & Decho (2001) Biofilms. Encyclopaedia of Life Sciences, Nature Publishing, London;  
30 <http://www.els.net>] known as biofilms, that are resistant to far higher concentrations of antibiotics than are required to kill individual cells [Chen et al (2002).

*Pseudomonas* infection; <http://www.emedicine.com/PED/topic2701.htm>; Qarah et al

(2001). *Pseudomonas aeruginosa* infections;  
<http://www.emedicine.com/MED/topic1943.htm>; Todar K (2002). *Todar's Online Textbook of Bacteriology: Pseudomonas aeruginosa*;  
<http://textbookofbacteriology.net/pseudomonas.html>; Iglewski BH (1996).

5 *Pseudomonas*. Medical Microbiology 4<sup>th</sup> edition, S. Baron (ed.). University of Texas; <http://gsbs.utmb.edu/microbook/ch027.htm>]. The practical effect of this is demonstrated by infections in cystic fibrosis patients, virtually all of whom eventually become infected with a bacterial strain that cannot be eradicated by the use of antibiotics, even when the isolated strain may appear to be sensitive in the  
10 laboratory [Høiby N (1998). *Pseudomonas* in cystic fibrosis: past, present, future. European Cystic Fibrosis Society Joseph Levy Memorial Lecture;  
[http://www.ecfsoc.org/pa\\_review/nh\\_lect.html](http://www.ecfsoc.org/pa_review/nh_lect.html)].

Only a few antibiotics are effective against *Pseudomonas aeruginosa*, including  
15 fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains. Multidrug resistance is common and increasing [Friedland I et al (2003). *Diagnostic Microbiology and Infectious Disease* 45:245-50; Henwood *et al* (2001). *Journal of Antimicrobial Chemotherapy* 47: 789-799]. The U.S. National Nosocomial Infections Surveillance System Report of June 1999  
20 [Gerberding J *et al* (2001). *National Nosocomial Infections Surveillance (NNIS) System Report*, data summary from January 1992-June 2001, issued August 2001. U.S. Department of Health and Human Services, Atlanta,  
[http://www.cdc.gov/ncidod/hip/NNIS/2001nnis\\_report.PDF](http://www.cdc.gov/ncidod/hip/NNIS/2001nnis_report.PDF)] states that antibiotic resistance of *Pseudomonas aeruginosa* isolated from nosocomial infections in ICU  
25 patients in 1999 had increased over the 1994-98 period for all classes of antibiotics studied.

There is therefore a demonstrated need for new approaches to the control of  
*Pseudomonas aeruginosa* infection.

30

Bacteriophages (often known simply as “phages”) are viruses that grow within bacteria. The name translates as “eaters of bacteria” and reflects the fact that as they grow, most bacteriophages kill the bacterial host as the next generation of

bacteriophages is released.

Early work with bacteriophages was hindered by many factors, one of which was the widespread belief that there was only one type of bacteriophage, a non-specific virus that killed all bacteria. In fact, the host range of bacteriophages (the spectrum of bacteria they are capable of infecting) is often very specific. This specificity may be considered a therapeutic strength as populations of bacteriophages can be selected to specifically eliminate only the target bacteria. Antibiotics, on the other hand, kill a wide range of bacteria and their use can consequently lead to disruption of the normal flora, leading to recolonisation of the body by inappropriate and often harmful bacteria.

Despite the therapeutic advantages afforded by the host specificity of bacteriophages, this characteristic has the disadvantage that it can be difficult to achieve breadth of coverage of target strains. Bacteriophage therapy has previously concentrated either on finding single bacteriophages to target specific bacteria, or on finding combinations of bacteriophages which are capable of targeting more than one bacterial species. In the former case, this has commonly involved the isolation of bacteria from the patient followed by the identification of specific bacteriophages which could be effective for that individual [Abul-Hassan et al (1990) *Annals of the MBC* 3: 262-265]. This is expensive and time-consuming, limiting the utility of the approach, especially in cases of critical illness.

## **Summary of the Invention**

Accordingly, the present invention resides in the use of a panel of bacteriophages which are all active against the same bacterial species, where the panel exhibits broader strain specificity for the target bacterial species than any of the individual bacteriophage components. The present invention therefore relates to the preparation, formulation and uses of such panels of bacteriophages.

In particular, the invention relates to combined preparations suitable for simultaneous or sequential use, comprising two or more bacteriophage strains, wherein each of said strains has activity against the same target bacterial species, and wherein the combination of said bacteriophage strains is capable of killing a greater number of strains of said target bacterial species than any one of the bacteriophage strains alone. In particular, the invention relates to such bacteriophage preparations for targeting *Pseudomonas aeruginosa* infections. Such a preparation may preferably be a single composition, for example a therapeutic composition.

More particularly, the invention provides eight bacteriophages deposited as NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, and mutants of any thereof which retain the ability to kill bacteria of the target species. One, two, three, four or more of said deposited strains may be used in the methods of the invention as described below.

The preparations of the invention are provided for use in a method of treatment of the human or animal body, by therapy. Also provided is the use of such preparations in the manufacture of a medicament for the treatment of bacterial disease, most especially *P. aeruginosa* infection, in humans or animals.

Also provided are combined preparations of (i) a preparation or strain of the invention and (ii) an alginase for simultaneous, separate or sequential use in the treatment of a human lung infection involving *Pseudomonas aeruginosa*.

The preparations of the invention may also be used in methods for detecting the presence of a target bacterial species. Accordingly, the invention provides a method of detecting the presence of the target bacterial species, for example *Pseudomonas aeruginosa*, in an *in vitro* sample, e.g. a biological sample from a human or animal for a diagnostic purpose, comprising contacting said sample with a preparation or strain of the invention, and determining whether said bacteriophage(s) are capable of

killing bacteria in said sample. Also provided is a kit for use in such a method, comprising a preparation or strain of the invention and means for detecting bacterial killing by said composition.

- 5 The invention also provides a method of identifying a bacterial strain indicative for an individual bacteriophage present in a therapeutic or other mixture, for example one of the eight deposited bacteriophages listed above, the method comprising the steps of measuring plaque formation by said bacteriophage in a number of bacterial strains; and selecting a strain which allows at least 1000 times more plaque formation  
10 by said bacteriophage than by the other bacteriophages in said mixture.

Also provided are bacterial strains identified or identifiable by such a method that are used to identify bacteriophages present in preparations intended for therapeutic use and/or to identify strains present in tissue samples obtained during such therapeutic  
15 use or following such use.

Such bacterial strains may also be used to determine the amount of a particular bacteriophage in a bacteriophage preparation, for example a preparation of the invention. Thus the invention provides a method of quantifying the amount of a  
20 bacteriophage in a preparation comprising two or more bacteriophages, the method comprising:

- (a) measuring plaque formation from said bacteriophage in a number of bacterial strains;
- (b) selecting a bacterial strain which allows at least 1000 times more plaque  
25 formation by said bacteriophage than by any of the other bacteriophages in said preparation; and
- (c) determining the concentration of bacteriophages in said mixture capable of growing on said bacterial strain.

# Brief Description of the Figures

Figure 1: Efficacy of bacteriophages against different strains of *Pseudomonas aeruginosa*. Strains named in bold were resistant.

- ☐ Plaques observed
- ☒ No plaques
- ☒ Either (1) Some dilutable inhibition observed but no obvious plaques, or  
(2) by visual assessment *P.aeruginosa* isolate deemed poorly susceptible
- ☐ ND Not done

5

The six bacteriophages BC-BP-01, BC-BP-02, BC-BP-03, BC-BP-04, BC-BP-05, and BC-BP-06 together resulted in 90% coverage of all screened *P. aeruginosa* strains.

10

Examples of bacterial isolates used:

Bacteria	Strain	Species origin	Date isolated	Location	Number of passages
7 <i>Used for BC-BP-04</i>	<i>Pseudomonas aeruginosa</i>	Human	1960's	US army surgical research unit Ft Sam Houston, Texas, USA	10-100
3708 <i>Used for BC-BP-01</i>	<i>Pseudomonas aeruginosa</i>	Human	1970's	Public Health Laboratory, Cambridge, UK	10-100
G184 <i>Used for BC-BP-02</i>	<i>Pseudomonas aeruginosa</i>	Human	1980's	Edinburgh, UK	10-100
919686 <i>Used for BC-BP-05</i>	<i>Pseudomonas aeruginosa</i>	Dog	1980's	Idexx Laboratories, Wetherby, UK	2-3
27225 <i>Used for BC-BP-06</i>	<i>Pseudomonas aeruginosa</i>	Dog	2003	Royal Veterinary College, London, UK	2-3
C33138 <i>Used for BC-BP-03</i>	<i>Pseudomonas aeruginosa</i>	Dog	2003	Axiom laboratories, Devon, UK	2-3

15

Figure 2: Identification of a BC-BP-03 count strain. Plates of count strain infected as follows:

A: Uninfected.

B: Infected with BC-BP-03 (1,000,000-fold dilution).

5 C: Infected with BC-BP-01 (10-fold dilution).

D: Infected with BC-BP-04 (10-fold dilution).

E: Infected with BC-BP-02 (10 fold dilution).

Figure 3: Resolution of infection in a dog ear treated with bacteriophage BC-BP-04:

10 A: Appearance of right ear 24 hours after treatment with 400 infectious units of BC-BP-04.

B: Appearance of left ear which did not receive bacteriophage treatment.

15 **Detailed Description of the Invention**

The present invention is based on the identification and development of panels of naturally-occurring viruses that infect pathogenic bacteria, and their formulation into therapeutic medicaments suitable for evaluation through the clinical trials process,  
20 permitting the use of these standardised panels against infections by the target bacteria either directly or following susceptibility testing.

The present invention therefore provides methods for the identification, characterisation, evaluation and development of panels of bacteriophages derived  
25 from environmental isolates. The invention provides combined preparations suitable for simultaneous or sequential use comprising panels of bacteriophages. A suitable preparation may therefore be a single therapeutic composition comprising a panel of the invention, or may comprise a number of separate compositions each comprising one or more bacteriophages from a panel of the invention, with the panel thus being  
30 split or spread between the separate compositions.

A suitable panel will consist of two or more strains of bacteriophage. For example, a suitable panel may consist of two or more, three or more, four or more, five or more,

or six or more bacteriophage strains. A preparation or composition of the invention may comprise two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty or more different bacteriophages. The bacteriophages may be from the same or different taxonomical groups of viruses.

5

Bacteriophages with the potential to control bacterial infections may be identified by a process of bioprospecting. This involves the identification of such agents by assay of material from sources rich in the target bacteria, and introduction of such material to cultures of the target bacteria. A suitable sample may be taken from sewage from  
10 a hospital, urban or other source.

Typically, sewage samples are mixed with powdered or liquid bacterial growth media and with the target strains of bacteria against which it is desired to isolate specific bacteriophages.

15

Samples are screened for the presence of suitable bacteriophages by monitoring their effect on bacterial cells. Typically this may involve determining bacterial death by observing the formation of cleared zones in bacteria grown on solid substrates ("plaques") or a loss of turbidity in liquid culture.

20

Each of the bacteriophage strains included in each of the preparations or compositions of the invention will have activity against the same target bacterial species. By activity is meant the ability of a bacteriophage to infect that bacterial species and to have a detrimental effect on the infected cells. This may be seen in  
25 the death of some or all of the infected cells. Preferably, infection with a bacteriophage of the invention will lead to lysis of some or all of the infected bacterial cells.

30

The bacteriophages present in a preparation of the invention will all have activity against the target bacterial species. Preferably, the activity of the bacteriophage will be specific to that bacterial species. That is, preferably the bacteriophages will have activity against the target bacterial species but will have no activity or lower activity against other bacterial species. For example, the bacteriophages may have the ability

to kill infected cells from the target species exclusively or at far higher efficiency than those of other bacterial species. Alternatively, the two or more bacteriophage strains of the preparation will have activity against the target species, but only one or none of these bacteriophages will have any demonstrable activity against any other  
5 bacterial species. Preferably the preparations of the invention will be inactive against any bacterial species other than the target species. The bacteriophages may have detectable activity (for example the ability to form plaques on bacteria cultured on solid media) against the target species at a concentration 1000 times or more lower than the concentration required to detect activity against another bacterial species.

10

Suitable bacteriophages may be from any of the families of viruses known to contain such bacteriophages, or may be from other virus families.

Once isolated, bacteriophages may be assayed against multiple strains (isolates) of  
15 the target bacterial species in order to determine their activity and specificity. These isolates may be taken from patients either infected or colonised with a bacterial species. Suitable isolates may also be obtained from natural or environmental sources of the bacterial target strain, such as soil samples. Methods of isolating bacteria from such samples are well known in the art. For example, suitable *P.*  
20 *aeruginosa* isolates for testing of bacteriophage panels may be obtained from known *P. aeruginosa* infections such as *otitis externa*, topical infections, burn infections, nosocomial infections, or other infections. Suitable isolates may also be obtained from natural sources of *P. aeruginosa*, such as soil samples.

25 The preparations of the invention exhibit a broader strain specificity for the target bacterial species than any of the individual bacteriophages in the preparation. That is, the preparation of bacteriophages is able to kill a greater number of strains or isolates of the target bacterial species than any of the individual bacteriophages that make up the preparation.

30

This may be achieved by including in the preparation a number of bacteriophage strains each having different specificities for the target bacterial isolates giving the preparation an overall total effectiveness against many more strains than any of the

individual bacteriophages. Additionally or alternatively, the preparation may include one or more bacteriophage strains which are effective against a broad spectrum of bacterial isolates of the target species so that the bacteriophages in the preparation have overlapping effectiveness, with some specific isolates being targeted by multiple bacteriophages in the preparation, thus helping to minimise any development of resistance. Individual strains of the target bacterial species may therefore be killed by one or more of the bacteriophages making up a preparation. This is exemplified by the development of a panel of bacteriophages effective against *Pseudomonas aeruginosa* shown in Figure 1.

The activity of bacteriophages against a range of isolates, for example at least 50 isolates, may be tested and the resulting information correlated to identify a group of at least two different bacteriophages which have a combined effectiveness against the target bacterial species that is greater than the effectiveness of any of the individual bacteriophages. These panels of bacteriophages then form the basis for the preparations of the invention.

Bacteriophages may be grown separately in strains (growth strains) of the host (or a related species) that support growth to high levels, titrated and combined at therapeutic concentrations. Typically, this may range from 100 to 100,000,000 infectious units per dose for each bacteriophage in the mixture.

In one embodiment the different bacteriophages are present in said preparation in substantially the same proportion, that is, substantially the same amount of each bacteriophage strain will be present in said preparation. In an alternative embodiment, the amount of each bacteriophage will depend upon the virulence of each bacteriophage strain against the target bacterial species. That is, a bacteriophage strain which is effective at a low concentration may be present in a lower amount and a bacteriophage strain that requires a higher titre for antibacterial activity will be present in a higher amount. In this way, a preparation that is consistently effective against the widest possible range of bacterial isolates may be achieved.

In one embodiment, the bacterial species targeted by a composition of the invention is *Pseudomonas aeruginosa*.

5 In particular, the present invention provides eight bacteriophage strains that are shown herein to be effective at killing a broad range of *P. aeruginosa* isolates. These bacteriophage strains were deposited at the National Collection of Industrial and Marine Bacteria (23 St Machar Drive, Aberdeen, AB24 3RY, Scotland, UK) on 24 June 2003 as follows:

10

Reference	NCIMB Deposit Number
BC-BP-01	NCIMB 41174
BC-BP-02	NCIMB 41175
BC-BP-03	NCIMB 41176
BC-BP-04	NCIMB 41177
BC-BP-05	NCIMB 41178
BC-BP-06	NCIMB 41179
BC-BP-07	NCIMB 41180
BC-BP-08	NCIMB 41181

A preparation of the invention may comprise one or more of these deposited bacteriophage strains. In one embodiment, the two or more bacteriophage strains of  
15 a preparation of the invention are all selected from these eight deposited strains. For example, a preparation may comprise any two, three, four, five six, or seven or all eight of these deposited strains. In another embodiment a preparation of the invention comprises two bacteriophage strains, both of which are selected from the eight deposited strains listed above. In a further embodiment, a preparation  
20 comprises the six strains BC-BP-01, BC-BP-02, BC-BP-03, BC-BP-04, BC-BP-05 and BC-BP-06.

In a related embodiment, any one of these eight deposited bacteriophages strains, or a mutant thereof, may be used in the methods of the invention. That is, the invention also provides compositions comprising one of these eight deposited strains as the sole bacteriophage. These compositions may be used in any of the therapeutic or  
5 diagnostic methods of the invention.

The target specificity of a bacteriophage may be altered by the choice of substrate on which it is grown. That is, two genetically identical bacteriophages may exhibit different target specificity when they have been grown on different substrates. In  
10 this case, a bacteriophage may be identified by the nucleotide sequence of its genome. A bacteriophage having the same genomic sequence as one of the eight deposited bacteriophage strains listed above is considered to be the same bacteriophage, even if the target specificity that it exhibits is not identical to that of the deposited strain.

15

It will be appreciated that the invention also extends to mutants of the deposited strains which retain the ability to kill bacteria of the target species. In particular, the invention extends to mutant forms of these strains which retain similar or improved target specificity as the strain from which they are derived. Thus, in a further  
20 embodiment, one or more bacteriophages in the preparations of the invention may be mutants derived from these deposited strains which retain the ability to infect and show activity against *Pseudomonas aeruginosa*.

Suitable mutant bacteriophages may be derived from one of the eight deposited  
25 strains BC-BP-01, BC-BP-02, BC-BP-03, BC-BP-04, BC-BP-05, BC-BP-06, BC-BP-07 and BC-BP-08. A suitable mutant strain may retain the ability to infect and kill the same target bacterial species as the deposited strain from which it is derived. For example, a mutant strain derived from one of the deposited strains listed above may retain the ability to infect and kill *P. aeruginosa* cells. A mutant strain may  
30 retain the same target specificity as the strain from which it is derived. That is, it may infect and kill the same isolates or strains of the target bacterial species as the deposited bacteriophage. Similarly, it may be ineffective against the same bacterial isolates or strains as the deposited bacteriophage. Alternatively, mutant

bacteriophage strains may be used which have altered target specificity, being more or less able to infect and kill particular isolates or strains of the bacterial target species.

5     Suitable mutant bacteriophage strains may retain a similar genome to a deposited strain. That is, the nucleotide sequence of the genome of a mutant bacteriophage may retain sequence identity to the genome of the deposited bacteriophage from which it is derived. Suitable mutant strains may retain at least 90%, at least 95%, at least 97%, at least 98% or at least 99% nucleotide sequence identity to the genome of  
10    a deposited strain, across the whole length of the genome. Alternatively, these levels of sequence identity may be retained across only a part of the genome, for example those parts of the genome required for target specificity. In one embodiment, the genome of a mutant bacteriophage may comprise a gene encoding a further therapeutic protein, as explained below. In such a case, the bacteriophage genome  
15    may consist of a genome having a degree of nucleotide sequence identity as set out above, plus those sequences necessary for the expression of the additional therapeutic protein.

The UWGCG Package provides the BESTFIT program which can be used to  
20    calculate sequence identity (for example used on its default settings) (Devereux *et al* (1984) Nucleic Acids Research 12, 387-395). The PILEUP and BLAST algorithms can alternatively be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S. F. *et al* (1990) J Mol Biol 215:403-10. Identity may  
25    therefore be calculated using the UWGCG package, using the BESTFIT program on its default settings. Alternatively, sequence identity can be calculated using the PILEUP or BLAST algorithms. BLAST may be used on its default settings.

Software for performing BLAST analyses is publicly available through the National  
30    Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length

in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

15 The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two polynucleotide or amino acid sequences would occur by chance.

20 For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

25 Mutations may be made to specific bacteriophages by chemical, radiological or other methods well known to those skilled in the art. Mutants with useful characteristics may then be selected by assay of infectious, physical or genetic characteristics, for example the ability to infect previously resistant bacterial strains. Mutation may also be made by homologous recombination methods well known to those skilled in the

30 art. The mutated sequence may comprise deletions, insertions or substitutions, all of which may be constructed by routine techniques. Insertions may include selectable marker genes, for example lacZ, for screening recombinant viruses by, for example,  $\beta$ -galactosidase activity.

Insertions may also include sequences that encode proteins desired for simultaneous administration with the bacteriophages, as described in more detail below. For example, one or more of the bacteriophages in a preparation of the invention may  
5 include a sequence encoding an alginase such that the alginase is expressed in an infected bacterial cell.

The bacteriophages may be combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents  
10 include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The bacteriophage preparation may be used directly, stored frozen in aqueous or  
15 other solution with an appropriate cryoprotectant (e.g. 10% sucrose), freeze dried and rehydrated prior to use, or rendered stable in some other formulation including (but not limited to) tablet, emulsion, ointment, or impregnated wound dressing or other item.

20 A preparation or strain of the invention may be used in a method of treatment of the human or animal body by therapy, in particular in the treatment of a condition related to an infection with the target bacterium. The invention therefore provides a method of treating a bacterial disease comprising administering to a human or animal in need thereof an effective amount of a preparation or strain of the invention. The  
25 preparations or strains of the invention may be used in the manufacture of one or more medicaments for the treatment of bacterial disease in humans or animals.

In particular, the preparations or strains of the invention may be used to address chronic or antibiotic-resistant infections. Thus, the use of bacteriophage preparations  
30 and strains as specified may initially be as a secondary treatment where infection is proving difficult to clear with existing antibiotics, or in combination or rotation where there is a critical need for clearance of infection. Thus, they may be used to complement and supplement antibiotic use.

In a preferred embodiment, the preparations and strains of the invention may be used for preventing or controlling infections caused by *Pseudomonas aeruginosa*.

5 The infection may be in a human or animal, for example a dog or a cat. The infection may be, for example, in or on the ear, eye, skin or other topical location. The infection may be systemic. *Pseudomonas aeruginosa* infections that may be treated by the methods of the invention include *otitis externa* and other ear  
10 infections, keratitis and other eye infections, folliculitis, infections of burns and induced graft rejection, wound infections, hospital acquired infections (nosocomial infections) and lung infections, for example in cystic fibrosis. Urinary tract infections and bacteraemias may also be treated.

The preparations of the invention may further comprise, or may be administered  
15 simultaneously, separately or sequentially with further therapeutic agents. For example, treatment with a preparation or strain of the invention may be coordinated with that of another agent relating to the condition being treated. The preparations or strains of the invention may be administered alongside antibiotics to complement or supplement their actions. The preparations or strains of the inventions may be  
20 administered alongside agents directed to other aspects of the condition of the patient, for example agents which may reduce inflammation, stimulate or reduce an immune response, relieve pain or otherwise improve the condition of the patient. The preparations or strains of the invention may be administered alongside other agents being used to treat a patient wherein the other agents may lead to an increased  
25 risk of bacterial (e.g. *Pseudomonas aeruginosa*) infection. For example, preparations or strains of the invention may be administered to a patient suffering from immunosuppression, such as localized immunosuppression due to treatment with another agent.

30 In one embodiment, the use of the preparations or strains of the invention to treat a *P. aeruginosa* infection, for example a lung infection, may be supplemented with the administration of an alginase. As explained above, *Pseudomonas* has a tendency to grow in a complex layer known as a biofilm. A biofilm is an assemblage of surface-

associated microbial cells that is enclosed in an extracellular polymeric substance (EPS) matrix. Alginate is the major component of the EPS matrix of *P. aeruginosa*. The use of alginase may therefore help to disrupt such *P. aeruginosa* biofilms and potentiate the clearance of infection. Biofilms may be present in a variety of *P.*

5 *aeruginosa* infections, including infections of the lung and ear. Co-administration of an alginase with a preparation or strain of the invention may be particularly suitable for use in treating such conditions.

The alginase may be included in the compositions of the invention. The alginase  
10 may be provided from a sequence within the genome of a bacteriophage in the preparation. This may involve the isolation of such bacteriophages from environmental sources or, for example, the genome of a bacteriophage may be engineered by methods known in the art to include such a sequence operably linked to suitable regulatory sequences. The alginase may be provided in a separate  
15 medicament which may be administered simultaneously with a preparation or strain of the invention, sequentially with a preparation or strain of the invention or separately to a preparation or strain of the invention.

The amount of bacteriophage administered will depend upon the size, location and  
20 nature of the area to be treated and the route of administration used. As a successful treatment will lead to multiplication of the bacteriophages and killing of infected bacteria, some treatments, for example those requiring topical infection, may require only a low dose of bacteriophages. This dose is measured in infectious units, usually defined by the ability to form cleared zones or "plaques" on bacterial culture plates.  
25 Such units are defined as "plaque forming units" or "pfu". For example, in some cases the dose may be a few hundred infectious units (pfu) or less. A suitable dose may be  $10^2$  to  $10^8$  pfu, preferably  $10^4$  to  $10^6$  pfu. In other cases, for example in a systemic or widespread infection, the dose may need to be higher to ensure that the bacteriophages reach all infected areas. In such a case a suitable dose may be in the  
30 range of from  $10^4$  to  $10^{10}$  pfu, preferably from  $10^5$  to  $10^8$  pfu. When injected, typically 10  $\mu$ l to 1 ml of bacteriophages in a pharmaceutically acceptable suitable carrier or diluent is administered. For topical administration the volume may be

higher, for example 100  $\mu$ l to 50 ml of the medicament, depending on the size, location and nature of the area to be treated.

5 Bacteriophage preparations and compositions of the invention may be administered to the human or animal patient topically, systemically, orally, or by some other means suitable for delivering an effective dose to the site of the infection to be treated.

10 The pharmaceutical composition is administered in such a way that the bacteriophage can be incorporated into bacteria at the site of infection. The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine the optimum route of administration and dosage for any particular patient and condition.

15 The preparations and strains of the invention may also be used in *in vitro* diagnostic methods to detect the presence of a specific bacterial species, for example *Pseudomonas aeruginosa*. Such methods may comprise the steps of contacting a test sample with a preparation or strain of the invention selected to be broadly effective against an individual bacterial species, and determining whether any of the  
20 bacteriophages thus added are capable of killing bacteria in the test sample. Preferably, the test sample will be cultured prior to contact with the preparation or strain, for example under conditions suitable to allow growth of any bacteria of the target species that are present. Suitable culture conditions are known in the art, and will depend upon the specific bacterial target species.

25

The preparations of the invention are particularly useful for such methods because they will detect a broad spectrum of bacterial strains or isolates. A single bacteriophage from a preparation of the invention may detect only a small proportion of strains or isolates of a particular bacterial species, and will therefore typically  
30 offer a very high false negative rate simply because of this high specificity. However, the use of a preparation of the invention, comprising two or more such bacteriophages, will allow detection of a broad spectrum of strains within a target bacterial species.

In one embodiment, the test sample may be cultured on a solid growth medium, such as on an agar plate. The sample is preferably cultured on said medium for a sufficient time and under suitable conditions for any target bacteria present in the sample to multiply on the surface of the plate. By contacting the surface of the plate with a preparation or strain of the invention, it can be determined whether any of the bacteriophages thus added are capable of infecting and killing the bacteria. The bacteriophage-infected medium may be maintained under suitable conditions for bacteriophage infection and replication, such that the bacteriophages have an opportunity to infect any target species (e.g. *P. aeruginosa*) cells on the plate. This will lead to the development of clear patches (plaques) where bacterial death has occurred, and will indicate that the test sample contained the target bacterial species.

In an alternative embodiment, the test sample may be maintained in a liquid medium. Again, it may be cultured under conditions suitable for bacterial growth. Following the addition of a preparation or strain of the invention, the medium may be maintained for a further period to allow the bacteriophages to infect any target bacteria present. This will lead to a loss of turbidity in the medium when bacterial death occurs, and this will indicate that the test sample contained the target bacterial species.

The test sample may be any sample where the presence of the target bacterial species is suspected. For example, the test sample may be from a source where *Pseudomonas aeruginosa* infection is suspected. The test sample may be from an environmental or biological source. In one embodiment, the test sample is from a human or animal patient who is suspected of having a bacterial infection. Such a test sample may be from, or derived from, a fluid or tissue sample obtained from the patient. The sample may be obtained from the location of an infection. In the case of a topical infection, the sample may be obtained by taking a swab from the infected region. The detection method of the invention may therefore be used to determine what species of bacteria is responsible for the infection, or to determine whether a particular bacterial species, for example *P. aeruginosa*, is involved.

An infection capable of being identified using a preparation or strain of the invention will normally be treatable using the same preparation or strain. That is, if the bacteriophages in a preparation of the invention are capable of killing bacteria obtained from the infected area *in vitro*, they should also be capable of killing the same bacteria *in situ* at the site of infection.

The detection method of the invention may therefore also be used to identify a suitable preparation or strain of the invention for use in treatment. The detection method of the invention may also be used to identify single bacteriophages that are suitable for use in such treatment individually, rather than in combination. That is, by using different bacteriophages or combinations of bacteriophages in the detection methods of the invention, the bacteriophage(s) with the greatest virulence towards the bacterial strain of the specific infection may be selected for use in treatment. Preferably, a combination of two or more bacteriophages having such activity is selected for use in treatment.

The present invention also includes the identification and use of "count strains", defined as strains of the target (or a related) bacterium which support the growth of one bacteriophage from a mixture of such bacteriophages, such as a preparation of the invention, while only permitting limited growth of all other bacteriophage components in the mixture. These count strains may then be used to assess titres of the bacteriophage stocks.

In particular, the invention provides count strains each of which support the growth of one of the eight deposited bacteriophages referred to herein as BC-BP-01 to BC-BP-08. The invention also provides a method for identifying a bacterial strain capable of acting as such a count strain, and uses of such count strains to identify bacteriophages in samples.

The preparations of the invention comprise at least two bacteriophages. Across a spectrum of bacterial strains, the growth of any one bacteriophage will be supported with varying efficiency (or not at all). Consequently, titres obtained by assaying across a range of bacterial strains will differ substantially. In order to provide a

means of determining/standardising the therapeutic dose to be administered to each patient, 'count' bacterial strains may be used. The principle of this approach is that the growth of each bacteriophage is only supported at a usable level by one of the range of bacterial strains. A count strain can thus be selected for each of the

5 bacteriophages in a mixture which supports the growth of one of the bacteriophages but does not support the growth, or only supports a low level of growth, of the other bacteriophages in the mixture. Titres of each bacteriophage constituent of the mixture may be calculated based on the growth possible on each of the individual count strains.

10

For example, a suitable count strain may allow at least 50 times more, at least 100 times more, at least 500 times more, at least 750 times more, at least 1000 times more, at least 1500 times more, at least 2000 times more growth, or greater, of one bacteriophage compared to the other bacteriophages being used in a mixture of

15 bacteriophages.

20

The differential growth may be assessed, for example by titrating plaque formation on the bacteria when grown on solid growth medium by the bacteriophages at a range of concentrations. Alternatively, the differential growth may be assessed by

looking at the size and nature of the plaques so formed. For example, in one embodiment, the count strain may allow at least 1000 times more plaque formation by one bacteriophage than other bacteriophages. Such a bacterium would form a count strain for that bacteriophage. For example, as shown in Figure 2, a suitable growth strain for BC-BP-03 may show significant plaque formation following

25 infection with BC-BP-03 at a 1,000,000-fold dilution, but little or no plaque formation when infected with other bacteriophages (here BC-BP-01, BC-BP-02 and BC-BP-04) at a 10-fold dilution (100,000 times higher concentration).

30

Accordingly, the invention provides a method of quantifying the amount of a bacteriophage in a preparation comprising two or more bacteriophages, for example a preparation of the invention, the method comprising:

- (a) measuring plaque formation from said bacteriophage in a number of bacterial strains;

- (b) selecting a bacterial strain which allows at least 1000 times more plaque formation by said bacteriophage than by any of the other bacteriophages in said preparation; and
- (c) determining the concentration of bacteriophages in said mixture capable of growing on said bacterial strain.

Such a count strain may also be used as a propagation strain for production of bacteriophage for use in the compositions of the invention. For example, a composition of the invention may be formed by combining the required count strains in the appropriate quantities. The count strains may therefore have a therapeutic use themselves as a source of bacteriophages.

This technique also enables the replication of each bacteriophage in a therapeutic composition to be monitored individually in a clinical context. Count strains specific for a particular bacteriophage may be used to identify the presence of that bacteriophage in particular, for example in preparations intended for therapeutic use or in tissue samples obtained during or following such therapeutic use. It is anticipated that this method will allow therapeutic bacteriophages to be distinguished from any extraneous bacteriophages that might exist in the strain with which the patient is infected, and would also allow the determination of which bacteriophages in the administered therapeutic mixture are active against that patient's bacterial infection. The 'count' bacterial strains can be used to 'type' any extraneous bacteriophage prior to administration of the bacteriophage therapy. The presence of the required bacteriophages in a composition or medicament may therefore be confirmed prior to treatment and the presence of the bacteriophages at the treatment site may be monitored during and after treatment. This information may be used by the medical practitioner to monitor and adjust the treatment regimen.

### Examples

#### *Isolation of bacteriophages active against Pseudomonas aeruginosa:*

- (i)  $3 \times 10^9$  colony forming units (cfu) of appropriate *Pseudomonas aeruginosa* strain cultured with settled sewage and nutrient broth (total volume 200ml).  
5
- (ii) Suspension incubated at 37°C for 24 hours.
- (iii) 1ml aliquot removed and filtered through 0.45µm syringe-top filter.
- (iv) Filtered lysate cultured with the same *Pseudomonas aeruginosa* strain used in step (i), and assessed for presence of bacteriophage (see below)
- 10 (v) Nutrient agar plates incubated at 37°C for 24 hours.
- (vi) Single plaque 'picked' using sterile 1mm diameter wire and used to inoculate 3ml of growth media (the constituents of this media varied between extractions) containing  $5 \times 10^6$  cfu/ml of the *Pseudomonas aeruginosa* strain used in step (i).
- (vii) Suspension incubated at 37°C until lysis of bacteria complete (this typically  
15 takes between 5-8 hours) and is assessed visually. The visual assessment is facilitated by comparing the turbidity of the bacteriophage-containing bacterial suspension with that of a control suspension. Control suspensions do not receive bacteriophage, yet are similar in every other respect.
- (viii) Lysate filtered through 0.1µm syringe-top filter.
- 20 (xi) Lysate adjusted to constitute 2% v/v glycerol, aliquotted into vials and stored at -80°C.
- (xii) Titres assessed by co-culturing with appropriate bacterial strain (see below)

#### *Preparation of Master Seeds:*

- 25 Master seed stocks are established for all bacteriophages as follows:
  - (i) Primary bacteriophage preparations were co-cultured with appropriate *Pseudomonas aeruginosa* propagating strain on agar plates (see below)

- (ii) Single plaques 'picked' using sterile 1mm diameter wire and used to inoculate 4ml of Vegetable Peptone Broth containing  $5 \times 10^6$  colony forming units (cfu)/ml of the *Pseudomonas aeruginosa* strain used in step (i).
- (iii) Suspension incubated at 37°C until lysis of bacteria complete (this typically takes between 5-8 hours) and is assessed visually. The visual assessment is facilitated by comparing the turbidity of the bacteriophage-containing bacterial suspension with that of a control suspension. Control suspensions do not receive bacteriophage, but are similar in every other respect.
- (iv) Lysate filtered through 0.1 µm syringe-top filter.
- (v) Master stock adjusted to constitute 2% v/v glycerol, aliquotted into vials and stored at -80°C.
- (vi) Titres assessed by co-culturing with appropriate bacterial strain (see below)

***Assessment of titres of individual bacteriophages within a mixed population:***

***Bacteriophage vs Pseudomonas aeruginosa 'count' bacterial strains***

- (i) Each bacteriophage (individual suspension) was assayed in duplicate on all count strains. The Master seeds were used.
  - (ii) Titres were calculated for each bacteriophage on each 'count' bacterial strain.
  - (iii) Steps (i)-to-(ii) were repeated on two further occasions.
  - (iv) On the final occasion the mixed bacteriophage suspension containing equal proportions of the 6 individual bacteriophage preparations was assayed in duplicate on all 'count' bacterial strains.
  - (v) Titres for bacteriophage mix on each 'count' bacterial strain were calculated
- All three sets of experiments yielded comparable results, which are detailed in Table 1, along with an averaged result

**Table 1a:** Experiment #1 – Bacteriophage vs ‘count’ bacterial strains

Titre (pfu/ml) (Titres presented are means of duplicate readings)

<i>P. aeruginosa</i> strain	Count 06	Count 02	Count 03	Count 01	Count 04	Count 05
Bacteriophage						
BC-BP-06	$5 \times 10^9$	-	-	-	-	-
BC-BP-04	-	-	Barely discernible inhibition. No plaques	-	$1.22 \times 10^8$	-
BC-BP-02	-	$2 \times 10^8$	Barely discernible inhibition. No plaques	-	-	-
BC-BP-05	-	-	-	-	-	$2.3 \times 10^9$
BC-BP-01	-	-	$6 \times 10^2$	$2.84 \times 10^8$	-	‘Turbulences’ observed at $10^{-1}$ and $10^{-2}$ Dilutes out
BC-BP-03	-	-	$3.1 \times 10^8$	$2.2 \times 10^3$	-	-

5

**Table 1b:** Bacteriophage vs cross-reacting ‘count’ bacterial strains; difference in titres (experiment #1)

10 Titre (pfu/ml) (Titres presented are means of duplicate readings)

<i>P. aeruginosa</i> strain	Count 03	Count 01	Fold difference
Bacteriophage			
BC-BP-01	$6 \times 10^2$	$2.84 \times 10^8$	$4.73 \times 10^5$
BC-BP-03	$3.1 \times 10^8$	$2.2 \times 10^3$	$1.4 \times 10^5$

**Table 1c:** Experiment # 2 – Bacteriophage vs 'count' bacterial strains

Titre (pfu/ml) (Titres presented are means of duplicate readings)

<i>P.aeruginosa</i> strain	Count 06	Count 02	Count 03	Count 01	Count 04	Count 05
Bacteriophage						
BC-BP-06	$4.8 \times 10^9$	-	-	-	-	-
BC-BP-04	-	-	Barely discernible inhibition. No plaques	-	$1.22 \times 10^8$	-
BC-BP-02	-	$7.5 \times 10^8$	Barely discernible. inhibition. No plaques	-	-	-
BC-BP-05	-	-	-	-	-	$1.58 \times 10^9$
BC-BP-01	-	-	$3.5 \times 10^2$	$8.1 \times 10^7$ (poor duplicates)	-	'Turbulences' observed at $10^{-1}$ and $10^{-2}$ Dilutes out
BC-BP-03	-	-	$2.65 \times 10^8$	$7 \times 10^2$	-	-

5

10

**Table 1d:** Bacteriophage vs cross-reacting 'count' bacterial strains; difference in titres (experiment #2)

Titre (pfu/ml) (Titres presented are means of duplicate readings)

<i>P. aeruginosa</i> strain	Count 03	Count 01	Fold difference
Bacteriophage			
BC-BP-01	$3.5 \times 10^2$	$8.1 \times 10^7$	$2.3 \times 10^5$
BC-BP-03	$2.65 \times 10^8$	$7 \times 10^2$	$3.8 \times 10^5$

15

**Table 1e:** Experiment #3 – Bacteriophage vs 'count' bacterial strains; individual and mixed.

Titre (pfu/ml) (Titres presented are means of duplicate readings)

Outcome of experiments where the 6 bacteriophages are mixed before assaying shown in **bold text**

<i>P.aeruginosa</i> strain	Count 06	Count 02	Count 03	Count 01	Count 04	Count 05
Bacteriophage						
BC-BP-06	<b>6.36x10<sup>9</sup></b> <b>5.8x10<sup>9</sup></b>	-	-	-	-	-
BC-BP-04	-	-	Barely discernible inhibition. No plaques	-	<b>4.62x10<sup>8</sup></b> <b>9.6x10<sup>8</sup></b>	-
BC-BP-02	-	<b>1.17x10<sup>9</sup></b> <b>7.5x10<sup>8</sup></b>	Barely discernible inhibition. No plaques	-	-	-
BC-BP-05	-	-	-	-	-	<b>1.53x10<sup>9</sup></b> <b>1.4x10<sup>9</sup></b>
BC-BP-01	-	-	1x10 <sup>2</sup>	<b>2.61x10<sup>8</sup></b> <b>3.3x10<sup>8</sup></b>	-	'Turbulences' observed at 10 <sup>-1</sup> and 10 <sup>-2</sup> Dilutes out
BC-BP-03	-	-	<b>3.69x10<sup>8</sup></b> <b>3.35x10<sup>8</sup></b>	7.5x10 <sup>2</sup>	-	-

10

**Table 1f:** Bacteriophage vs cross-reacting 'count' bacterial strains; difference in titres (experiment #3)

Titre (pfu/ml) (Titres presented are means of duplicate readings)

<i>P. aeruginosa</i> Strain	Count 03	Count 01	Fold difference
Bacteriophage			
BC-BP-01	1x10 <sup>2</sup>	<b>3.3x10<sup>8</sup></b>	3.3x10 <sup>6</sup>
BC-BP-03	<b>3.35x10<sup>8</sup></b>	7.5x10 <sup>2</sup>	4.5x10 <sup>5</sup>

15

**Table 1g:** Bacteriophage vs 'count' bacterial strains; mean results of experiments #1, #2 and #3

Titre (pfu/ml) (Titres presented are means of duplicate readings in experiments #1, #2 and #3)

5

<i>P.aeruginosa</i> strain	Count 06	Count 02	Count 03	Count 01	Count 04	Count 05
Bacteriophage						
BC-BP-06	$5.2 \times 10^9$	-	-	-	-	-
BC-BP-04	-	-	Barely discernible inhibition. No plaques	-	$4.8 \times 10^8$	-
BC-BP-02	-	$5.3 \times 10^8$	Barely discernible inhibition. No plaques	-	-	-
BC-BP-05	-	-	-	-	-	$1.8 \times 10^9$
BC-BP-01	-	-	$3.5 \times 10^2$	$2.3 \times 10^8$	-	'Turbulences' observed at $10^{-1}$ and $10^{-2}$ Dilutes out
BC-BP-03	-	-	$3 \times 10^8$	$1.2 \times 10^3$	-	-

10 **Table 1h:** Bacteriophage vs cross-reacting 'count' bacterial strains; difference in titres. Means of experiments #1, #2 and #3

Titre (pfu/ml) (Titres presented are means of duplicate readings in experiments #1, #2 and #3)

<i>P. aeruginosa</i> strain	Count 03	Count 01	Fold difference
Bacteriophage			
BC-BP-01	$3.5 \times 10^2$	$2.3 \times 10^8$	$6.6 \times 10^5$
BC-BP-03	$3 \times 10^8$	$1.2 \times 10^3$	$2.5 \times 10^5$

15

***Preparation of Purified Bacteriophage Suspensions:***

Bacteriophages are prepared for use from master suspensions as follows:

- (i) 30ml suspensions of appropriate growth strains of *Pseudomonas aeruginosa*  
5 in Vegetable Peptone Broth inoculated with master seeds of the appropriate bacteriophage at a multiplicity of infection of 0.1.
- (ii) Suspension incubated at 37°C until lysis of bacteria complete (this typically takes between 5-8 hours) and is assessed visually. The visual assessment is facilitated by comparing the turbidity of the bacteriophage-containing bacterial suspension with  
10 that of a control suspension. Control suspensions do not receive bacteriophage, but are similar in every other respect.
- (iii) Sub-master seeds filtered through 0.45µm then 0.1µm syringe-top filters.
- (iv) 27 ml of the sub-master seeds carefully over-layered onto 5ml of a 10% w/v sucrose 'cushion', in 36ml polypropylene centrifuge tubes. The purpose of the  
15 sucrose 'cushion' is to prevent the sedimentation of endotoxins, while allowing the virus particles to pellet at the bottom of the tube.
- (v) All centrifuge tubes and buckets thoroughly cleaned and then autoclaved at 121°C prior to use.
- (vi) Tubes spun at 23,500 rpm at 4°C for 3 hours in a Beckman ultra-centrifuge
- 20 (vii) Supernatant fractions removed and the pellets drained. Pellets then re-suspended in 1ml PBS+10% v/v glycerol and filtered through 0.2µm syringe-top filters.
- (vi) Titres assessed by co-culturing with appropriate bacterial strain (see below)
- 25 This material can be used *in vivo*, subject to sterility controls:

***Sterility***

The final product was tested for sterility as follows:

- (i) Three 0.6ml aliquots of final therapeutic product randomly selected

- (ii) Each aliquot spread out on nutrient agar plate (permissive for growth of a range of bacterial species including *Pseudomonas*) using sterile wire loop
- (iii) Plates incubated at 37°C for 48 hours
- (iv) Plates checked for presence of bacterial colonies

5

*(Such tests on the material as prepared showed no bacterial growth)*

***Assessment of efficacy:***

For the bacteriophage product designed to combat *Pseudomonas aeruginosa* strains which cause canine *otitis externa*, the development of the product entailed selection of appropriate bacteriophages to fulfil this role. This was achieved by co-culturing a range of 22 bacteriophages with 100 clinical *Pseudomonas aeruginosa* isolates derived from canine *otitis externa* infections as indicated below. It was found that 90% of these strains were susceptible to at least one of the 6 of the candidate bacteriophages BC-BP-01 to BC-BP-06 (Figure 1) from the initial panel of 22 strains. These 6 bacteriophages were progressed into clinical trials on the basis that this *in vitro* data supported the expectation that the product will be clinically effective *in vivo*.

20    **Method**

- (i) Bacteriophage preparation diluted in PBS in a 10-fold dilution series at room temperature.
- (ii) 100µl of appropriate dilution(s) added to 2.5ml molten agar at 46°C containing  $5 \times 10^6$  cfu of relevant bacteria.
- 25    (iii) Molten agar suspensions poured onto nutrient agar plates and allowed to set at room temperature.
- (iv) Plates transferred to 37°C and incubated for 24 hours.
- (v) Plaques counted and numbers used to calculate titre in pfu/ml. One is aiming to count the dilution which gives around 100 plaques per plate.

***Assessment of safety:***

A veterinary clinical trial was conducted to assess the toxicity of the bacteriophage product. The total duration of the study was 21 days. Six dogs (3 male; 3 female) received the following treatment regimen administered aurally on 3 occasions, at days 0, 3 and 6 of the study:

Group	Number of animals	Sex		Treatment	Dose Volume Left Ear	Dose Volume Right Ear
1 (control)	2	1 Male	1 Female	Diluent	0.2ml	0.2ml
2 (test)	4	2 Male	2 Female	Bacteriophage	0.2ml (10x therapeutic dose)	0.2ml (100x therapeutic dose)

10

Administration of the treatment was made by drops of liquid suspension into the external ear canal which was then massaged to promote deep penetration.

15

The following investigations were undertaken during the study:

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25

(i) Microbiological flora at days 0, 3 and 6 (samples taken immediately prior to administration of treatment) were assessed by plating of ear swabs on:

- 1) Cetrimide agar, selective for *Pseudomonas* spp
- 2) Mannitol salt agar, selective for *Staphylococcus* spp
- 3) Sabouraud dextrose agar, selective for yeast and moulds
- 4) FP agar, selective for micrococci
- 5) Blood agar, nonselective permitting growth of most microorganisms

(ii) Auroscopic veterinary examination daily until day 8 of the study, then every three days until the conclusion of the study

(iii) Core temperature measured daily

Throughout the study:

(i) ---Auroscopic veterinary examination revealed no significant changes in the condition of the ears of dogs in the test group, as compared with baseline recordings and the control group.

(ii) There were no significant changes in the microbiological flora within the ears  
5 of test group dogs, as compared with baseline recordings and the control group.

(iii) In the test group, core temperature recordings did not differ significantly from those noted in the control group or at baseline.

***Assessment of clinical efficacy:***

10 Studies to date have been based on the use of single bacteriophages of proven effectiveness, and the panel as selected is based on this work

***Protection of mice from lethal infection with Pseudomonas aeruginosa***

1. 150,000,000 infectious units (10 LD<sub>50</sub>) of *Pseudomonas aeruginosa* were  
15 injected into the peritoneal cavities of 20 mice

2. Groups of 5 mice were treated with 4 different concentrations of bacteriophage BC-BP-08, administered simultaneously with the bacterial injection

20 **Table 2:** Mouse survival after 150,000,000 infectious units (10 LD<sub>50</sub>) of *Pseudomonas aeruginosa* were injected into the peritoneal cavities of 20 mice

Bacteriophage (infectious units)	Not surviving	Surviving
290,000,000	0	5
29,000,000	4	1
5,800,000	5	0
290,000	5	0

***Prevention of the destruction of pig skin in vitro by Pseudomonas aeruginosa***

25 Fourteen wound models were made each consisting of four layers of enzyme cleaned, sterilized, freeze dried pig dermis soaked in human plasma. 100,000 cfu of

*P. aeruginosa* were placed on the top of each. Seven of the wound models received 1,000,000 infectious units of phage BC-BP-08, the remainder serving as controls. After 18 hours incubation, 7 of 8 of the controls were blindly assessed as decayed, whereas all of the phage treated models were assessed as not decayed. Bacteria  
5 could only be detected in three of the treated discs (median count 0; highest value 12,000). Phages were found to have penetrated through to the bottom layer of the model and have replicated in it (median count in models after incubation : 32,000,000; range 14,000,000-20,000,000,000).

10 ***Protection of skin grafts on guinea pigs from infection with Pseudomonas aeruginosa***

1. A 0.2mm thick rectangle of shaved skin (2cm x 1cm) was excised from the backs of 14 guinea pigs
2. The underlying skin layers were removed to make the wounds comparable to  
15 an excised burn
3. 600,000 infectious units of *Pseudomonas aeruginosa* were introduced into the wounds
4. 12,000,000 infectious units of the bacteriophage BC-BP-08 were introduced into the wounds on 7 animals, with no bacteriophage introduced into the  
20 wounds of the other 7
5. The skin rectangle was replaced and dressed
6. Graft success was assessed after 5 days

25 **Table 3: Protection of skin grafts on guinea pigs from infection with *Pseudomonas aeruginosa***

	Graft succeeded	Graft failed
No bacteriophage	0	7
With bacteriophage	6	1

***Multiplication of administered Pseudomonas aeruginosa bacteriophages in the external auditory canal of a dog with otitis externa***

The dog, with a history of atopy, had bilateral chronic otitis externa. Swabs of both ears had repeatedly grown *P. aeruginosa* for many months, despite antibiotic  
5 therapy. Before phage therapy, the dog had bilateral swollen erythematous external auditory canals; each with a purulent discharge and copious waxy secretions over the surrounding pinna. Swabs taken from each ear at that time grew as *P. aeruginosa* (identified by API 20NE, Biomerieux, France). The isolates from the two ears had differed slightly in their biochemical reactions and in their antibiotic sensitivity  
10 patterns. From a phage collection, eight were selected for testing against the strains, because they had previously been identified as exhibiting good lytic activity against a wide range of *P. aeruginosa* strains. Three of the phages displayed good lytic activity against both isolates obtained from the dog. The phages were further tested for how few plaque-forming units would lyse a standard broth cultures of the two  
15 isolates. BC-BP-04, the phage for which the number of pfu required for lysis was lowest was chosen for *in vivo* work. The phage concentration in the stock solution had been titrated, with 0.2 ml of a  $10^5$  dilution of this solution being applied by syringe to the dog's right external auditory meatus. This volume contained approximately 400 infectious units (plaque forming units, p.f.u.).

20

Twenty seven hours after phage application, a swab was taken of the detritus within the right ear. It was weighed before and after swabbing and the mass of detritus was determined. Phages were counted in the detritus by plating serial 10 fold dilutions using the agar overlay technique. There were  $1.6 \times 10^8$  in the 0.032g of detritus that  
25 was present on the swab, so it is likely that the phage multiplied over 1 million fold. This increase was accompanied by a marked clinical improvement of the right ear. There was less inflammation; disappearance of the purulent discharge; and reduced amount of waxy secretions (Figure 3a). The appearance of the left ear, which had not received phage, remained unchanged (Figure 3b). In view of the change, 400 pfu of  
30 the phage was applied to the left ear, which was followed by considerable clinical improvement 24 - 48 hours later. Two weeks after the phage application, the dog's ears had deteriorated; and swabs of both were positive for *P. aeruginosa* on culture. Subsequently the condition of the ears repeatedly deteriorated and improved for

many months the owner and veterinarian judged that their condition was better than it had been before phage was administered. Nine months after phage administration both ears completely recovered, and *P. aeruginosa* has not since been isolated from aural swabs. No antibiotics were given to the dog after phage administration as they  
5 were not considered necessary.

*Use of a Pseudomonas aeruginosa bacteriophage in the treatment of an infection of a human burn*

A single case trial was carried out on a 27 year old man with 50% burns. Episodes of  
10 healing of the burns alternated with periods of breakdown of the skin. It was noted that the skin on his back and chest were breaking down. At this time the clinicians, who were concerned by the rapid rate of breakdown of the skin, asked if a phage could be found to treat his *P. aeruginosa* infection. A new phage, BC-BP-07, was isolated that was active. Although *in vitro* testing did not indicate great activity  
15 against the strain, time was limited, so it was selected for further work. A purified suspension was made and no evidence of toxicity of which was shown when it was added to cultures of human epidermal cells. Approximately 1000 infectious units (p.f.u.) of BC-BP-07 were applied to each of two filter paper discs of diameter 25 mm. At a dressing change these were placed on areas of the patient known to be  
20 colonised with *P. aeruginosa*. 48 hours later counts of phage in the discs were  $1.2 \times 10^6$  and  $4.3 \times 10^4$ , increases of 1,200x and 43x. After this the patient's burns were sprayed with the phage. Following this the patient's condition gradually improved and he survived and eventually all wounds healed over. Whether the phage contributed to the recovery of the patient, who was also given antibiotics, is  
25 unknown, but phage did multiply on the burns and demonstrates the bacteriophage multiplying in or on a patient, thus indicating killing of bacteria by the bacteriophage.

Prior to their deposit at NCIMB on 24 June 2003, none of the bacteriophages referred  
30 to herein as BC-BP-01 to BC-BP-08 were publicly available, hence any reference to such strains in any publication or other disclosure before that date does not represent enabling prior art.

CLAIMS

1. A combined preparation suitable for simultaneous or sequential use, comprising two or more bacteriophage strains wherein each of said strains has activity against the same target bacterial species, and wherein the combination of said bacteriophage strains is capable of killing a greater number of strains of said target bacterial species than any one of the bacteriophage strains alone.
2. A preparation according to claim 1 which is a single therapeutic composition.
3. A preparation according to claim 1 or 2 which comprises three or more, preferably four or more, of said bacteriophage strains.
4. A preparation according to any one of the preceding claims wherein each of said bacteriophage strains has a different strain specificity for the target bacterial species.
5. A preparation according to any one of the preceding claims wherein the target bacterial species is *Pseudomonas aeruginosa*.
6. A preparation according to any one of the preceding claims wherein one or more of said bacteriophage strains are selected from NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or mutants of any thereof which retain the ability to kill *P. aeruginosa*.
7. A preparation according to claim 6 wherein said composition comprises two or more bacteriophage strains and wherein said strains are selected from NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or mutants of any thereof which retain the ability to kill *P. aeruginosa*.

8. A preparation according to claim 6 comprising three or more, preferably four or more, bacteriophage strains selected from NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or mutants of any thereof which retain the ability to kill *P. aeruginosa*.

9. A preparation according to claim 6 comprising the bacteriophage strains NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178 and NCIMB 41179, optionally supplemented by one or both of the bacteriophage strains NCIMB 41180 and NCIMB 41181, deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or mutants of any thereof which retain the ability to kill *P. aeruginosa*.

10. A preparation according to any one of claims 6 to 9 wherein said mutant strain(s) has at least 95% nucleotide sequence identity over the length of the entire genome to the genome of said deposited bacteriophage(s).

11. A bacteriophage strain selected from NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or a mutant of any thereof which retains the ability to kill *P. aeruginosa*.

12. A bacteriophage strain according to claim 11 which is a mutant strain having at least 95% nucleotide sequence identity over the length of the entire genome to the genome of said deposited bacteriophage.

13. A preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12 for use in a method of treatment of the human or animal body by therapy.

14. A preparation according to claim 9 for use in a method of treatment of canine *otitis externa* infection.

15. The bacteriophage strain NCIMB 41177 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, for use in a method of treatment of canine *otitis externa* infection.

16. The bacteriophage strain NCIMB 41180 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, for use in a method of treatment of *Pseudomona aeruginosa* infection associated with burns.

17. Use of a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12 in the manufacture of a medicament for the treatment of bacterial disease in humans or in animals.

18. Use according to claim 17 wherein said disease is a chronic or antibiotic resistant bacterial infection.

19. Use according to claim 17 or 18 wherein said bacterial disease is caused by *Pseudomonas aeruginosa* infection.

20. Use according to claim 19 wherein the animal to be treated is a dog or cat.

21. Use according to claim 20 wherein the infection is of the ear or eye or other topical location.

22. Use according to claim 19 wherein treatment is of a human infection involving *Pseudomonas aeruginosa*.

23. Use according to claim 19 wherein said infection is a nosocomial infection.

24. Use according to claim 22 or 23 wherein the infection is of the ear, eye, skin or other topical location.

25. Use according to claim 24 where the infection is associated with a burn.

26. Use according to claim 24 wherein treatment is of a human lung infection.

27. Use according to claim 26 wherein said lung infection is associated with cystic fibrosis.

28. Use according to any one of claims 19 to 27 where said medicament further comprises an alginase.

29. Use according to claim 28 where the alginase is expressed from a sequence present within the genome of one or more of the bacteriophages present in said preparation.

30. Use of (i) a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12 and (ii) an alginase in the manufacture of a medicament for simultaneous, separate or sequential use in the treatment of an infection involving *Pseudomonas aeruginosa*.

31. A combined preparation comprising a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12 and an alginase for simultaneous, separate or sequential use in the treatment of an infection involving *Pseudomonas aeruginosa*.

32. A method of treating a bacterial disease comprising administering to a human or animal in need thereof an effective amount of a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12.

33. A method of detecting the presence of a target bacterial species in an *in vitro* sample, comprising contacting said sample with a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12, and determining whether said bacteriophage are capable of killing bacteria in said sample.

34. A method according to claim 33 comprising culturing said sample on a solid growth medium, contacting said culture with said preparation or strain, and detecting any plaque formation on said solid medium, the presence of plaque formation indicating that said sample contained the target bacterial species.

35. A method according to claim 33 comprising culturing said sample in a liquid medium, contacting said culture with said preparation or strain, and detecting

any loss of turbidity in said liquid medium, a loss of turbidity indicating that said sample contained the target bacterial species.

36. A method according to any one of claims 33 to 35 wherein said sample is from a human or animal.

37. A kit for use in a method of any one of claims 33 to 36, comprising a preparation according to any one of claims 1 to 10 or a strain according to claim 11 or 12 and means for detecting bacterial killing by said preparation or strain.

38. A method of identifying a bacterial strain selective for one of the bacteriophages NCIMB 41174, NCIMB 41175, NCIMB 41176, NCIMB 41177, NCIMB 41178, NCIMB 41179, NCIMB 41180 and NCIMB 41181 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, or mutants of any thereof which retain the ability to kill *P. aeruginosa*, the method comprising the steps of measuring plaque formation from said bacteriophage in a number of bacterial strains; and selecting a strain which allows at least 1000 times more plaque formation by said bacteriophage than by any of said other bacteriophages.

39. A bacterial strain identified or identifiable by a method of claim 38.

40. A bacterial strain identified or identifiable by a method of claim 38 for use in a method of treatment or diagnosis of the human or animal body.

41. Use of one or more bacterial strains identified or identifiable by a method of claim 38 to identify and/or quantify bacteriophages present in preparations intended for therapeutic use and/or to identify strains present in tissue samples obtained during such therapeutic use or following such use.

42. A method of quantifying the amount of a bacteriophage in a preparation comprising two or more bacteriophages, the method comprising:

(a) measuring plaque formation from said bacteriophage in a number of bacterial strains;

(b) selecting a bacterial strain which allows at least 1000 times more plaque formation by said bacteriophage than by any of the other bacteriophages in said preparation; and

(c) determining the concentration of bacteriophages in said mixture capable of growing on said bacterial strain.

43. A method according to claim 42 wherein said preparation is defined as in any one of claims 1 to 10.

44. Use according to claim 42 or 43 wherein said preparation is a therapeutic mixture.

45. Use according to claim 41 or a method according to claim 42, 43 or 44 wherein the bacterial strains are strains of *Pseudomonas aeruginosa*.

**ABSTRACT**

The present invention relates to a combined preparation suitable for simultaneous or sequential use, comprising two or more bacteriophage strains wherein each of said bacteriophage strains has activity against the same bacterial species, and wherein the  
5 combination of said bacteriophages is capable of killing a greater number of strains of said target bacterial species than any one of the bacteriophage strains alone.

The invention also includes the preparation, assay, and formulation of such preparations active against *Pseudomonas aeruginosa*, and the use of such a mixture  
10 against infections of humans or animals which involve *Pseudomonas aeruginosa* including topical, nosocomial and lung infections.

The invention also includes the use of "count strains" of host bacteria which show exclusive sensitivity to one of the strains present in a therapeutic or other mixture.

Strain	920361	923883	925255	924212	925250	925327	922481	922895	920482	921101	925494	920097	919686	920710	925880	27191	27731	27036	27115	27209	26056	WOO340	925576	926032	WOO133
BACTERIOPHAGE																									
BC-BP-01								X				X													
BC-BP-04			X					X																	
BC-BP-02												X								X					
BC-BP-05			X																						
BC-BP-06										X						X									
BC-BP-03																									
BC-BP-08								X			X														
BC-BP-07																									

Strain	925984	925617	WOO331	WOO341	924903	925480	925738	924226	WOO339	925295	WOO334	921513	WOO132	C33199	C32417	C33139	C33051	C33391	918672	918625	918682	918040	918884	918701	28471
BACTERIOPHAGE																									
BC-BP-01		X							X																
BC-BP-04		X																					X		
BC-BP-02																									
BC-BP-05																									
BC-BP-06					X																				
BC-BP-03		X																							
BC-BP-08									X														X		
BC-BP-07								X																	

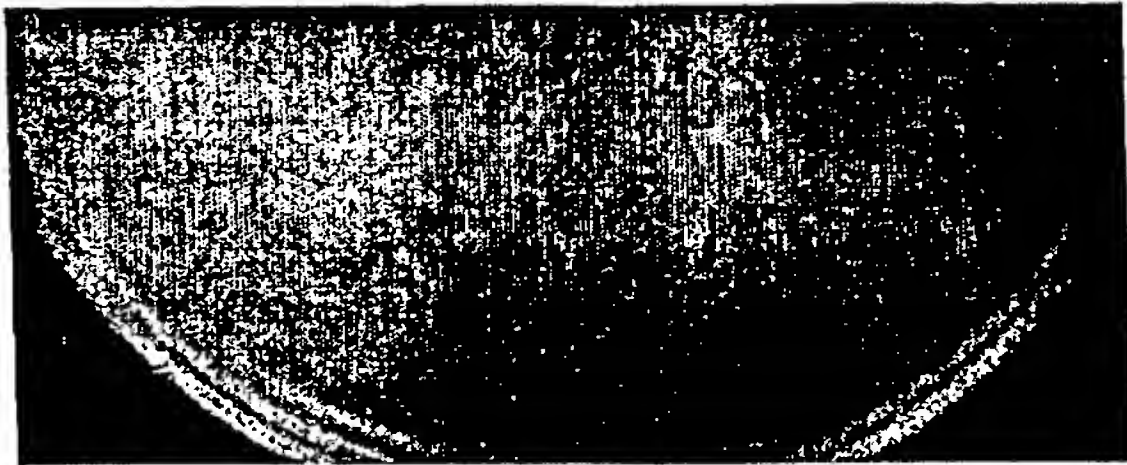
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BACTERIOPHAGE																									
BC-BP-01																									
BC-BP-04							X									X									
BC-BP-02																									
BC-BP-05																									
BC-BP-06																									
BC-BP-03											X														
BC-BP-08																X									
BC-BP-07																									X

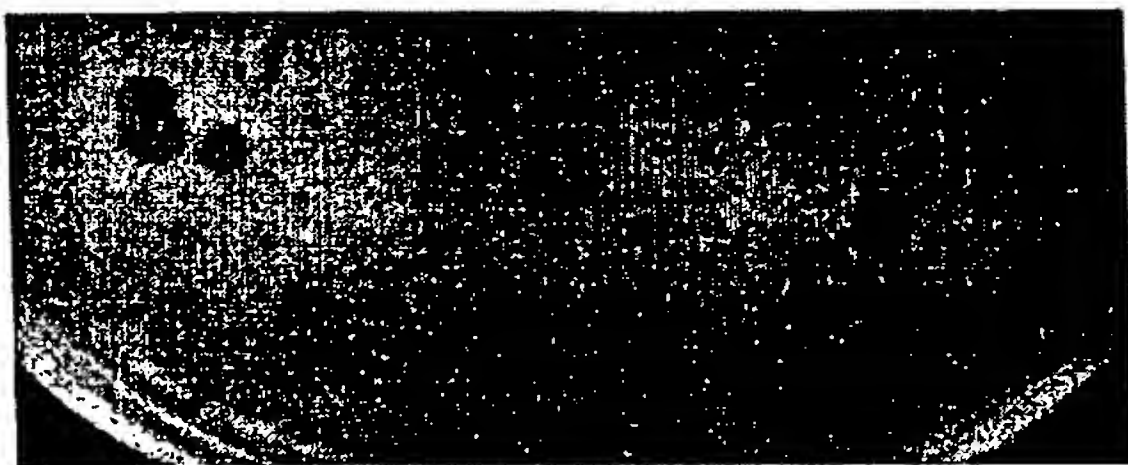
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BACTERIOPHAGE																									
BC-BP-01					X			X		X													X		
BC-BP-04							X	X		X									X						
BC-BP-02					X		X	X													X				
BC-BP-05																									X
BC-BP-06																			X		X	X			
BC-BP-03																				X					
BC-BP-08						ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BC-BP-07						ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Figure 1

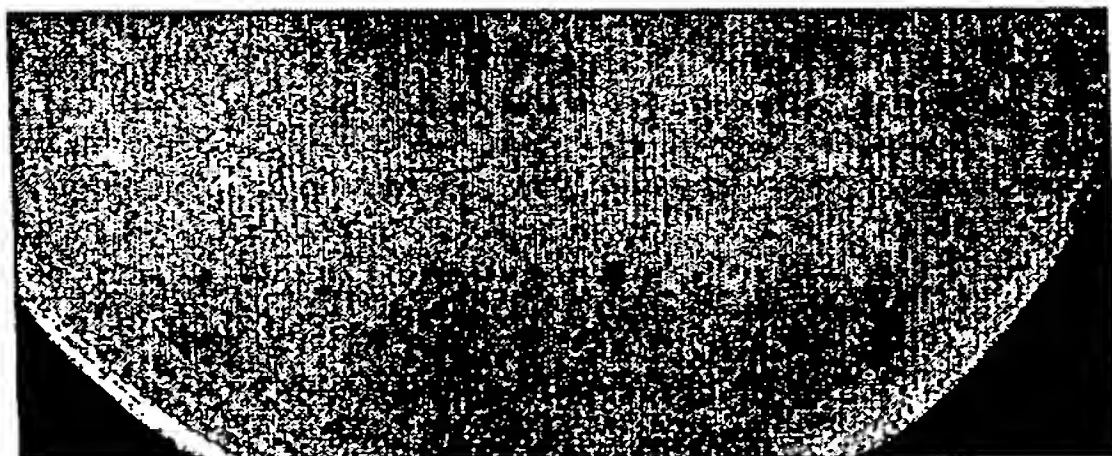
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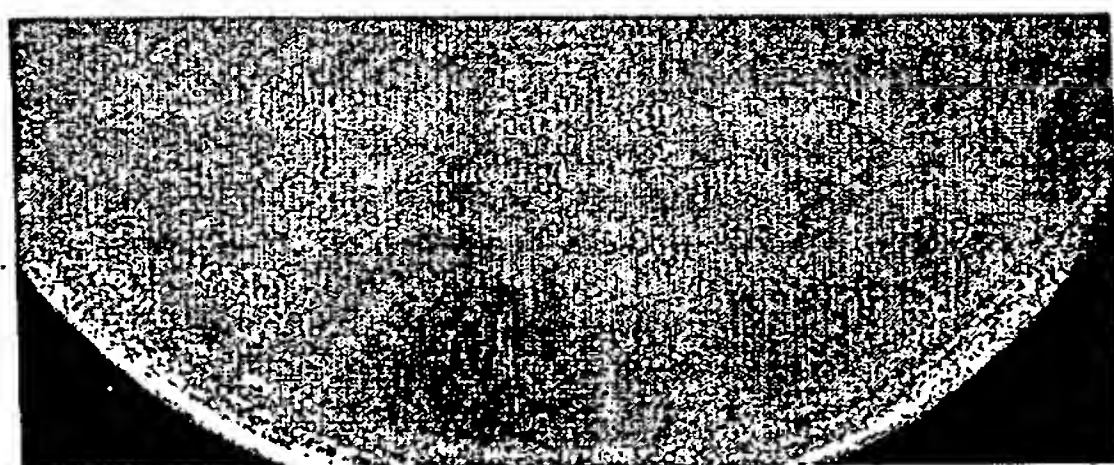
**B**



**C**



**D**



**E**

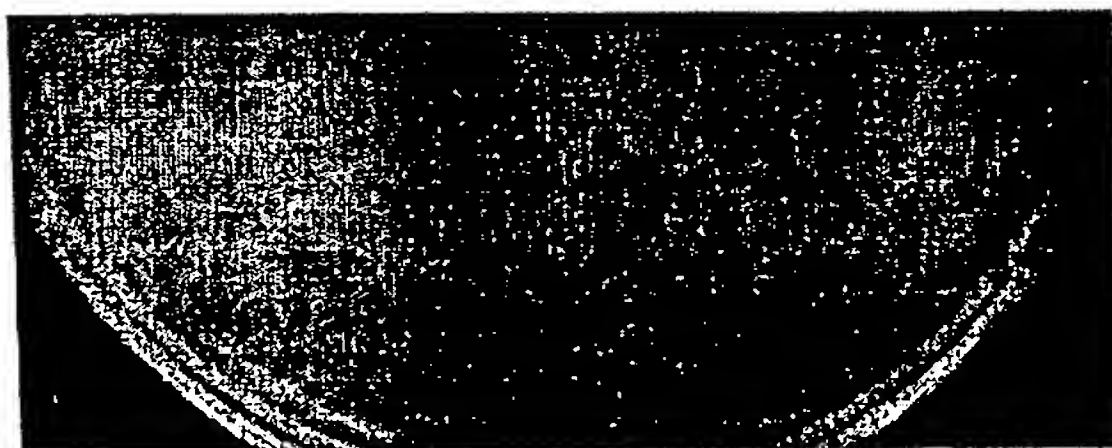


Figure 2

A



B



Figure 3

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